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A new factor influencing pathogen detection by molecular assay in children with both mild and severe hand, foot, and mouth disease☆

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ABSTRACT

This study aimed to find novel information concerning pathogen detection and some probable coinfection factors in hand, foot, and mouth disease (HFMD). In this study, 1104 clinically diagnosed HFMD patients were included. Enterovirus 71 (EV71), coxsackievirus A16 (CA16), and 14 different respiratory pathogens were examined from nasopharyngeal swabs using polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR). To evaluate the immune activation in HFMD patients, 8 cytokines and IgM antibodies to EV71 and CA16 from mild and severe patients were detected. Our results indicated that the severity of HFMD may affect the pathogen detection. The lower positive rates of enterovirus and respiratory viruses in severe HFMD cases by RT-PCR were probably related to stronger immune response. Therefore, immunological tests such as ELISA are essential supplements to PCR or RT-PCR in order to increase pathogen diagnosis in HFMD, especially in severe cases.

1. Introduction

Hand, foot, and mouth disease (HFMD) is generally a febrile exanthematous disease mostly prevalent in children younger than 10 years of age. Manifestations are vesicles on the palmar and plantar surfaces of the hands and feet, and on the buccal mucosa, tongue, and buttocks. Although other types of enterovirus (EV), such as coxsackie virus A4–A7, B2–B5, and EV18 are related to HFMD, human EV71 and coxsackievirus A16 (CA16) are the main pathogens (Bruu, 2003). Severe cases with neurological and cardiopulmonary complications, such as aseptic meningitis, encephalitis, and poliomyelitis-like paralysis, are mainly caused by EV71 (Chong et al., 2003; McMinn et al., 2001; Wang et al., 1999, Weng et al., 2010). In addition, EV71 is associated with higher mortality rates.

The early diagnosis of HFMD relies on typical clinical manifestations, together with RT-PCR assays from nasopharyngeal swab, vesicular fluid, or feces, with a detection rate of various EV that ranges from 48.0% to 88.4% (Chen et al., 2009; Jiang et al., 2012; Singh et al., 2002; Yang et al., 2011). It has been reported that different types of sample, early or multiple sampling may affect the positive rate of RT-PCR (Singh et al., 2002). Immune detection of IgM antibody against EV71 or CA16 using serum samples, which is meaningful for early diagnosis, has been established (Hong et al., 2012; Xu et al., 2010). However, the performance of the methods had not been fully evaluated. Recent studies have indicated that some antiviral proinflammatory and inflammatory cytokines were increasing after EV infection, especially in severe HFMD cases, and have been associated with certain adverse outcomes (Chung et al., 2008; Khong et al., 2011; Lin et al., 2002). The strong or abnormal activation of the immune system in severe HFMD cases may affect the method choice concerning pathogen detection using either molecular methods or immune detection focus on IgM antibody.

On the other hand, it has been reported that various viruses, such as human adenovirus (HAdV), norovirus, and rotavirus, were related to fatal HFMD cases (Cardosa et al., 1999; Liu et al., 2012). A broader insight into other common community-acquired pathogens (CAP) such as respiratory viruses (RV), *Chlamydophila pneumoniae* (CP), and *Mycoplasma pneumoniae* (MP) coinfection is necessary for understanding the pathogenesis and better management of HFMD. Laboratory detection of respiratory pathogens using molecular diagnostic tests such as RT-PCR have been frequently used in febrile children with respiratory symptoms, whereas the method and detection rate still need additional evaluation, especially in the situation when there is a coinfection with EV.

☆ The current "Instructions to Authors" have been read, and we declare to comply with the instructions and stated conditions. All authors and acknowledged parties agreed to the submitted version, and agreed to our inclusion. The material is original and it has been neither published elsewhere nor submitted for publication simultaneously. If accepted, the paper will not be published elsewhere.

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In this study, EV71, CA16, and 14 respiratory pathogens were examined using nasopharyngeal swabs from individuals with HFMD using commercially available PCR or RT-PCR kits. In addition, eight proinflammatory and inflammatory cytokines and the IgM antibodies released in both EV71 and CA16 infection were detected using serum samples from selected mild and severe HFMD cases. Our study yielded novel information concerning the pathogen detection in HFMD cases.

2. Materials and methods

2.1. Clinical specimen and data collection

After obtaining approval from the Ethics Committee of Beijing Youan Hospital, Beijing, China and consent from the study participants, a total of 1,104 clinically diagnosed HFMD patients were included in the study. Participants attended Beijing Youan Hospital between June and October in 2010 comprised 233 severe cases and 871 mild cases, with the mean age at 2.26, and 60.4% being male. Of the 233 severe cases, the mean age was 1.91, and 67.8% were male. Of the 871 mild cases, the mean age was 2.24, and 58.4% were male. All the patients were given a clinical diagnosis of HFMD by senior physicians with special training, and in accordance with the HFMD clinical diagnosis guidelines published in 2009 by the Chinese Ministry of Health. According to the guidelines, the mild cases were characterized by fever or not, with herpetic stomatitis and a rash on the hands and feet. The cases with any neurological signs and cardiopulmonary complications, such as aseptic meningitis, encephalitis, and poliomyelitis-like paralysis, were defined as severe cases.

Nasopharyngeal swabs from all the 1104 patients were collected within 5 days of the onset of illness (each patient sampled only once). The specimens were immediately placed in virus transport media tubes (YOCON, Beijing, China), which were temporarily stored at 4 °C for analysis or at −80 °C within 24 hours until use. Nasopharyngeal swabs from 348 cases, with 115 randomly selected mild cases as the mild group and 233 severe cases as the severe group, were chosen for CAP detection. Simultaneously, 103 serum samples were collected from the 348 patients for the immunological assay, with 33 samples from mild cases and 70 from severe cases. Serum samples were collected at the same time as nasopharyngeal swabs and stored at −80 °C until use.

2.2. Total RNA extraction and reverse transcription

RNA was extracted using QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany), according to the protocol provided. Reverse transcription was carried out using Revert Aid First Strand cDNA Synthesis Kits (Fermentas, Shenzhen, China) following the procedure outlined by the manufacturer.

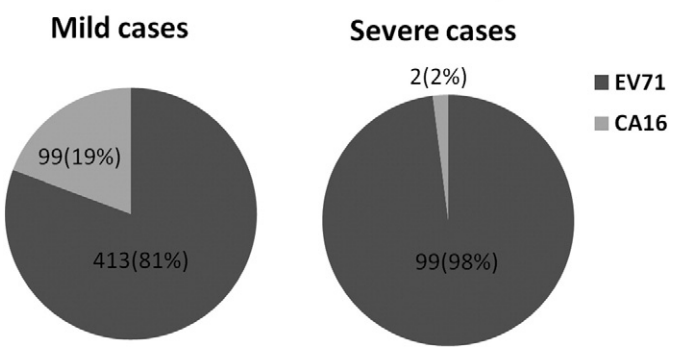

Fig. 1. The proportion of EV71 and CA16 in severe and mild HFMD cases. EV71 indicates enterovirus 71; CA16, coxsackievirus A16. Among the severe cases positive for EV71/CA16, 99 (98.0%) cases were positive for EV71, and only 2 were positive for CA16. On the other hand, in the mild cases positive for EV71/CA16, 413 (81%) cases were positives for EV71, and 99 (19%) were positive for CA16. The result indicates that EV71 is the major pathogen in HFMD patients, especially in severe cases.

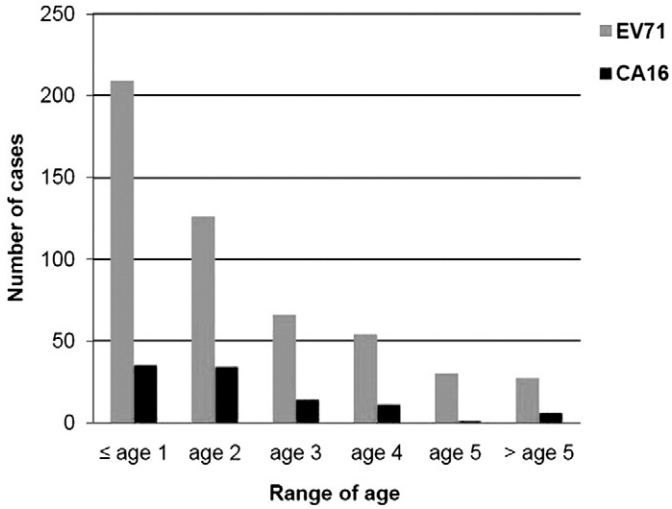

Fig. 2. The age profile of 611 cases positive for EV71/CA16. The majority (94.9%) of the patients were children younger than 5 years.

2.3. EV71, CA16 and human enterovirus universal (EVU) RT-PCR assays

EV71 and CA16 were analyzed using one-step RT-PCR Detection Kits (Da An Gene Co. Ltd., Guangzhou, China) in 1104 HFMD cases. Overall, 137 hospitalized cases negative for EV71 and CA16 were investigated for other EV infections using Human EVU Fluorescence RT-PCR Diagnostic Kits (Da An Gene Co. Ltd., Guangzhou, China). RT-PCR amplification was carried out using the M×3000P PCR instrument (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions.

2.4. Multiplex RT-PCR detection for RV

In 348 swabs chosen for CAP detection, multiplex RT-PCR was performed using Seeplex RV12 ACE Detection Kits (Seegene, Hangzhou, China) for RV detection, following the manufacturer's instructions. cDNA samples were used for the virus testing, and each cDNA sample was tested against 2 sets of primers: one set was tested for human HAdV; human metapneumovirus; human coronavirus

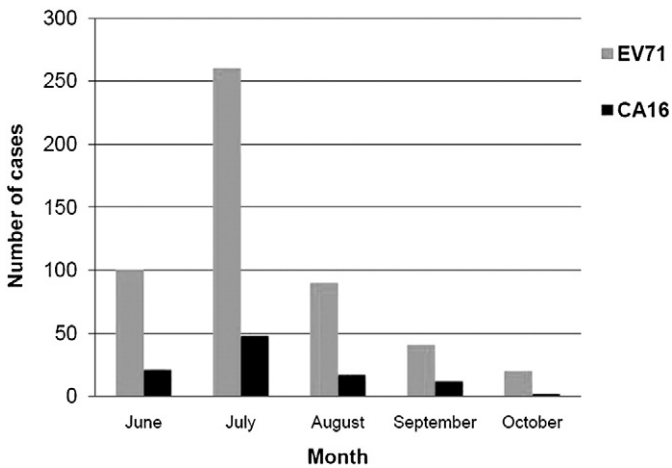

Fig. 3. Monthly distribution of the cases positive for EV71/CA16. The majority of the patients positive for EV71/CA16 were during the period from June to August. The peak incidence occurred in July. Both EV71 and CA16 positive patients were in accord with this trend generally.